STUDYING SPACE EFFECTS ON MICROORGANISMS AUTONOMOUSLY: GENESAT, PHARMASAT, AND THE FUTURE OF BIO-NANOSATELLITES

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ABSTRACT

The high cost, long wait times, and overall inaccessibility of the Space Shuttle and ISS for life sciences experiments in space has created a need for more accessible and lower cost alternatives. The purpose of the GeneSat-1 mission was to validate low-cost "cubesat" satellites as a novel platform for space life sciences research. GeneSat-1 was an autonomous, free-flying space satellite that proved capable of supporting microorganism growth and monitoring gene expression via fluorescence measurements of GFP promoter reporter constructs. Temperature, pressure, relative humidity, radiation events and acceleration in three axes were also monitored and reported by multiple sensors. Researchers with GFP promoter reporters for their genes of interest can readily use the GeneSat platform to test the effects of the space environment on the expression of these genes. Furthermore, the GeneSat hardware was used as a basis from which to add features and elements of interest, resulting in PharmaSat, the first principal investigator-driven cubesat biology mission. PharmaSat is scheduled to launch in September, 2008 and possesses increased complexity (optical measurements at 3 wavelengths, more fluidic capabilities) and sample number. The PharmaSat fluidic system comprises 59 sample wells, 48 of which are plumbed to support biology, including two media exchanges and the growth-triggered addition of a pharmacological agent at multiple concentration levels. The platform will enable monitoring of the action of an antifungal agent having potential countermeasure (therapeutic) application against the yeast *Saccharomyces cerevisiae*.

KEY WORDS: Gene expression, microgravity, nano-satellites, small satellites, microfluidics

INTRODUCTION

Spaceflight affects complex organisms in a number of ways, including increased immune stress, decreased bone density, muscle atrophy, and slowed wound healing. Microorganisms and mammalian cells in culture have also shown differences attributable to spaceflight, including altered growth rates. The study of spaceflight effects on cells has been limited by high costs and limited flight opportunities. Our program develops integrated platforms for economical, frequent space access for autonomous biological studies of the molecular details of such effects on microorganisms.

Several groups have built and flown integrated bioreactors aboard manned space vehicles (Cefai et al., 1994; Hammond et al., 1999; Walther et al., 1994). However, these systems required a human operator for initiating the experiment, recording data, collecting samples for analysis, and/or freezing or fixing samples for return to Earth for analysis (Hammond et al., 1999).

Small satellites like GeneSat can fly as "secondary" payloads: they "hitch a ride" into orbit with a much larger primary satellite. This "hitchhiker" approach to space science offers much lower launch costs than dedicated space biology and manned missions, but can add operational constraints and challenges such as the lack of access for late loading or re-loading of biological samples, and absence of power for thermal control and in-situ monitoring during the pre-launch phase.

We present here the details of the GeneSat-1 mission, including its purpose, the hardware developed to support that purpose, the experiment designed to demonstrate the technology, and the results from that experiment. We also introduce the hardware upgrades made to the GeneSat system in support of PharmaSat—a principal investigator (PI)-driven experiment that will result in publication-quality data.

GeneSat Hardware Design

Limitations and Capabilities

GeneSat, a small satellite configuration measuring 10 x 10 x 30 cm^3 and conforming to the so-called "3-U format", each "U" being a 10-cm cube [http://cubesat.atl.calpoly.edu/pages/home/background.php], relies on a number of innovations and compromises in order to conform to multiple constraints. A satellite of this size cannot offer sample return and, as such, must be fully autonomous. Also, as a secondary payload, the satellite and its experiment are inaccessible once integrated with the spacecraft for most flight opportunities, including TacSat-2, the host mission for GeneSat-1. Late loads and re-loads due to launch slips are typically not possible. For GeneSat-1, integration occurred 4 weeks before the scheduled launch, hence all organisms and reagents had to survive for six weeks before experiment initiation: four weeks of prelaunch activity, with two weeks of reserve in case of launch delays. Data transmission and communication with the satellite are possible only during a few several-minute passes per day over one or more pre-arranged ground stations, limiting the possibilities of human control of satellite subsystems.

Because of these limitations, GeneSat was designed for fully autonomous operations, including initiating the experiment, controlling the temperature, feeding the cells, and measuring, recording, and telemetering all data. To monitor the status of the environment in which the biology experiment was housed, GeneSat-1 used a number of small sensors within the payload pressure vessel: six for temperature (Analog Devices AD 590), one for pressure (Motorola MPXH6101A), one for relative humidity (Sensirion SHT15), three for acceleration (Silicon Designs 1221-002), and one for radiation (Hamamatsu PIN diode, S3071).

GeneSat Fluidic Card

The GeneSat fluidic card includes ten 110-µL culture wells and two solid-state reference wells. The fluidic card is made of multiple layers of laser-cut acrylic laminated together with pressure-sensitive-adhesive with the wells spaced such that they can be analyzed using standard multiwell plate readers. There are 0.5 µm nylon filters placed on the entrance and exit of each well to keep cells from escaping. A layer of optical-quality acrylic on the bottom of the fluidic card and a clear gas-permeable membrane on top enable high-quality optical measurements and gas exchange for each well. Figure 1 shows a cross section of a GeneSat fluidic card well.

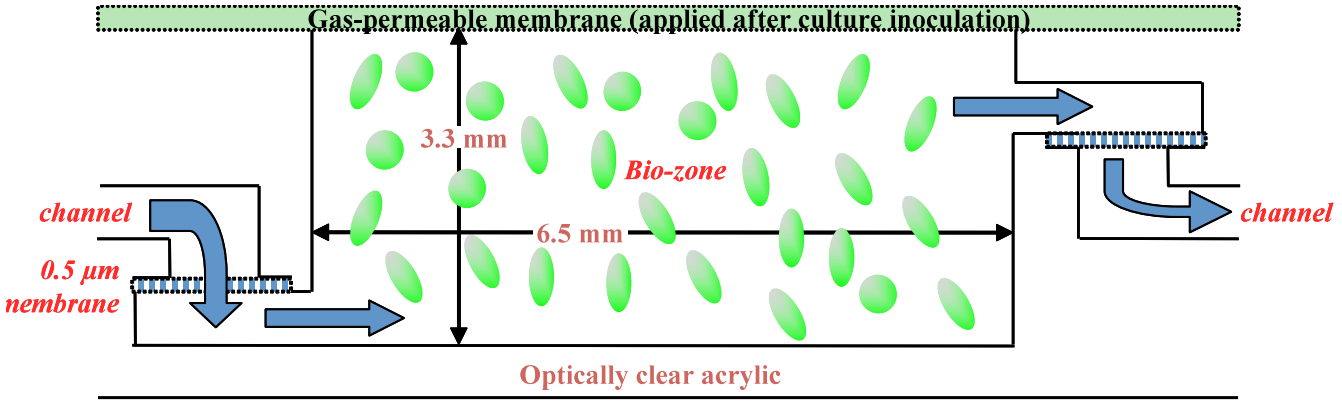

Figure 1: *Cross section of sample well in GeneSat fluidic card*

Initially, cells are loaded in the wells in stasis buffer to place them in a state of metabolic dormancy. Once the experiment is initiated (after attainment of stable orbit), growth medium is introduced to the wells, replacing the stasis medium and initiating cell growth.

GeneSat Fluidic System

The simple GeneSat fluidic system consists of two medical-grade polyethylene vinyl acetate bags under compression by a spring-backed metal plate; the output of the nutrient bag is connected to the fluidic card via medical-grade tubing and a normally-closed solenoid valve (Parker). When the experiment is initiated, the valve opens and the cells are fed as the pressure on the medium bag causes flow through connecting tubing into the fluidic card, displacing the stasis medium. The second bag serves two functions. It is connected directly to the outlet of the fluidic card without a valve and contains stasis medium at low pressure (3.5 kPa) to replace any buffer lost by evaporation through the gas-permeable well membranes during the stasis period, minimizing pre-flight air bubble formation in the wells. When the experiment is initiated, the evaporation-control bag becomes a waste bag: the higher pressure (30 kPa) in the nutrient bag pushes the stasis medium out of the wells and into the waste bag.

GeneSat Optics System

To avoid alignment issues due to moving parts, 12 separate optical units were used, one for each well. Each unit was designed to measure both the fluorescence and optical density. To measure fluorescence, a blue LED (470 nm; Luxeon) is focused to a ~ 3 mm spot inside the well via a pair of lenses and an excitation filter (Chroma) and is detected via an emission filter (525 nm, Chroma) and a pair of lenses to collect and focus green fluorescence on an intensity-to-frequency detector (TAOS TSL 237). To measure optical density, a green LED (Osram; driven at 2.3 mW) is illuminated and the same detection optics measure culture light scattering, an indirect measure of optical density, which is also directly proportional to cell number. This optics system, shown in Figure 2, allows fluorescence to be related to scattering, normalizing for culture population and providing gene expression on a per-organism basis.

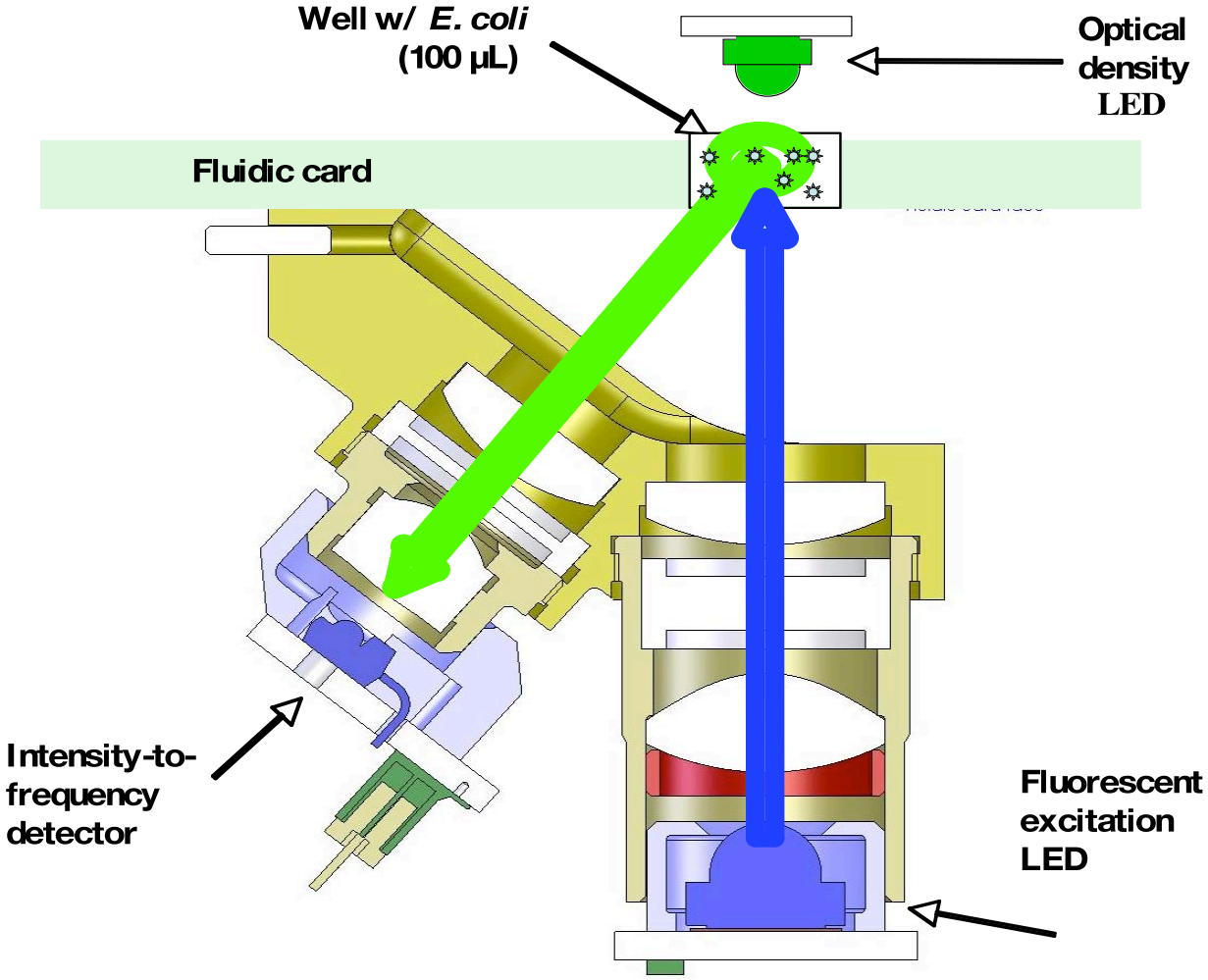

Figure 2: *GeneSat optical system diagram*

MATERIALS AND METHODS

Strains and Media

The medium used for growing cells both in flight and for ground testing was Luria Broth (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl; Sigma). Stasis buffer consisted of phosphate-buffered saline (PBS; Fisher Scientific) and saline solution (0.5% NaCl; Sigma). The LB used for flight was supplemented with 2.4 mg/mL carbenicillin antibiotic (Sigma) and the PBS and saline solutions were supplemented with 0.1 mg/mL carbenicillin for plasmid maintenance. Media used for growing the strains in the lab were supplemented with either carbenicillin or ampicillin (Sigma) for plasmid maintenance.

The following two *E.coli* strains, both expressing GFP constitutively, were used for flight: strain MM294 containing the pGREEN plasmid (Carolina Biological Supply Co.) and DH5α cells containing the plasmid AcGFP (Clontech).

Ground Testing

Different *E. coli* strains were compared as to their survival and GFP expression. Survival was assessed by growing cells to stationary or mid-log phase in LB, then storing them for long periods of time in either PBS or 0.5% saline. Percent survival of the different strains and conditions was assessed by colony counts on LB plates containing 0.1 mg/ml ampicillin.

Strains were also compared for their overall GFP expression and the stability of the GFP expression over time when stored in stasis conditions. GFP fluorescence was measured using a SpecraMAX GeminiXS fluorometer (Molecular Devices).

Fluidic Card Loading

To prepare cells for loading into fluidic cards, they were grown in LB containing 0.05 mg/ml carbenicillin. The DH5α/AcGFP strain was grown overnight while the MM294/pGREEN strain was grown to mid-log phase, both cultures were then harvested via centrifugation and resuspended in PBS containing carbenicillin.

Fluidic cards were fabricated (ALine) without a cover layer over the wells, sterilized using ethylene oxide, then stored in sterile bags until loading. To prepare a card for laboratory or flight measurements, solid-state reference

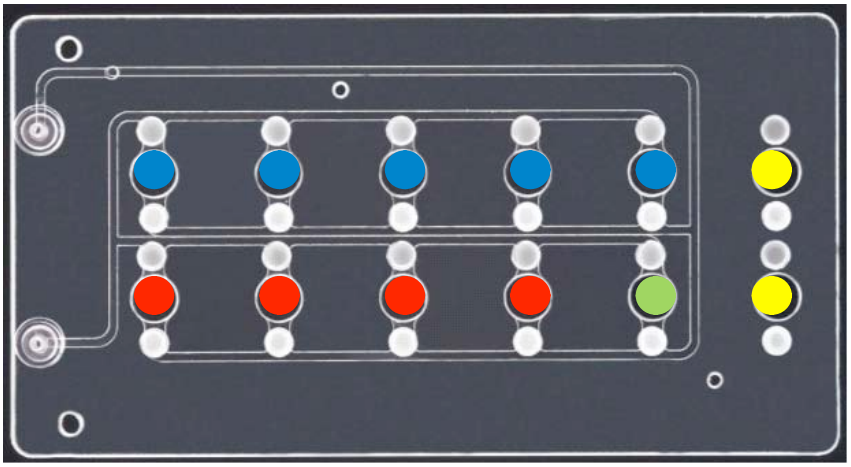

Figure 3: *Configuration of loaded strains in GeneSat fluidic card. Blue wells denote wells loaded with MM294/pGREEN strain, Red wells denote wells loaded with DH5α/AcGFP strain, and the yellow well was not loaded with cells (negative control).*

standards (Matech) are placed in the two "dry" wells. Each biowell was then inoculated with 10 µL of dormant *E. coli* culture in PBS with carbenicillin antibiotic (0.1 mg/mL). To help eliminate gas bubbles, wells and channels were purged with CO_2 gas. The open wells were then covered by a sterile, 75-µm-thick gas-permeable polystyrene (PS) membrane. Channels and inoculated biowells were next filled with helium-sparged 0.5% saline solution with 0.1 mg/mL carbenicillin; any CO_2 bubbles dissolved gradually as saline stasis medium flowed through.

Flight Conditions

For flight, two fluidics cards were loaded at NASA Ames Research Center (ARC) as depicted in Figure 3, five wells with pGREEN (shown in blue) and 4 wells with AcGFP (shown in red). One well was not loaded with cells and served as a negative control (shown in green). The two wells shown in yellow housed the fluorescent reference standards. The two cards were loaded within a day of each other. These fluidic cards were loaded into two identical satellite systems that were subsequently hand-carried to Wallops Field for final testing. Engineering functional checkouts were run on both systems and one was selected for integration while the other was hand-carried back to ARC to serve as the ground control.

Results

Ground Testing and Strain Selection

Because of the long lag time between the loading of the biology into the hardware and launch, ground testing needed to include the effects of this extended storage period on both the biology and the reagents. Ground testing prior to flight consisted of strain selection, hardware biocompatibility testing, stasis buffer selection, and reagent stability testing. Selection of an appropriate strain was dependent on the stability and level of GFP expression as well as the long-term survival in stasis buffer of the strain. The ideal strain would be hardy and express GFP consistently and at high levels—even after the stress of being exposed to long-term stasis with no nutrients.

The biocompatibility of the selected strains with all hardware materials was proved by determining: 1) that the cells in the hardware grew in a manner comparable to that in standard laboratory equipment, and 2) that cells would survive after long-term storage in contact with the fluidic card materials. The long-term stability of all the reagents was also tested.

A number of different *E. coli* strains was tested and two were selected for flight, MM294 (pGREEN plasmid) and DH5α cells (plasmid AcGFP). Both strains expressed GFP constitutively and at high levels. The MM294/pGREEN strain had more stable GFP expression but there was no information available on the promoter, so effects from microgravity could not be ruled out. The DH5α/AcGFP had higher GFP expression but this expression would sometimes vary when the cells were exposed to stress. Additionally, this second strain had a mild biocompatibility problem with one of the materials in the payload pressure vessel. Choosing to fly two strains reduced the chances that the experiment would fail due to unexpected factors.

Flight Results

On December 16, 2006, the GeneSat-1 satellite launched aboard a Minotaur I rocket from the Wallops Flight Facility in Virginia, USA as a secondary payload, TacSat-2 being primary. Less than 25 minutes after launch, GeneSat-1 was ejected into low-Earth orbit as a free-flying, autonomous satellite. Communications with the satellite were established on Dec 17, 2006. Some 49 h after launch, with two-way radio communication well established and GeneSat-1 responding to commands, the command was sent to initiate the experiment. Due to launch slips, the experiment was started some 48 days—just under 7 weeks—after the bio-load.

Maintaining stable temperature is one of the more important science requirements for most biology experiments. Figure 4 shows actual temperature data from GeneSat-1 in Earth orbit. After about one hour, the fluidic card reached the target 34 ºC and remained stable at that temperature throughout the duration of the experiment.

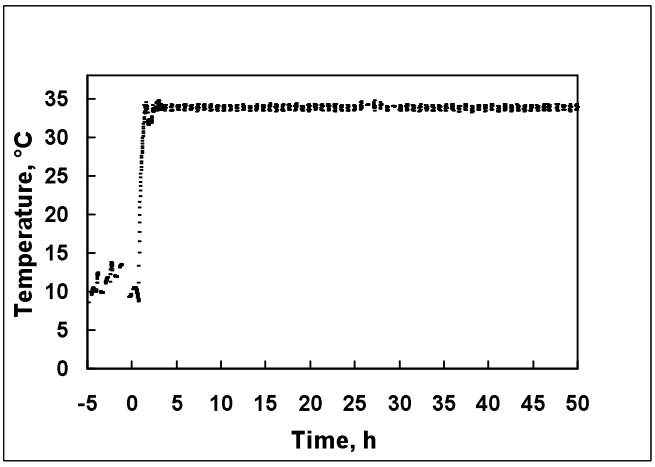

Figure 4: *GeneSat-1 temperature data. Actual temperature profile from GeneSat-1 flight. Time=0 denotes experiment start.*

After ~ 1 h at the 34 °C setpoint, the valve opened automatically, introducing nutrient (LB with 2.4 mg/mL carbenicillin) to displace the stasis buffer and start culture growth. *E. coli* in several wells exhibited measurable growth 3 h after nutrient addition and by 10 h after growth medium introduction, all 9 biowells had measurable growth. The two different *E. coli* strains had different initiation times and initial growth rates, as expected. One of the AcGFP wells was very slow to start growing; this was expected and likely due to the stress caused by the extended pre-launch stasis time. Figure 5 shows the optical density and growth inside both the flight and ground units and Figure 6 shows the fluorescent readings. pGREEN wells are shown in shades of blue, AcGFP wells are shown in shades of red, and the control wells are shown in green.

The doubling times for the different strains were calculated and the results are shown on Figure 7. A significant difference was observed between the ground and flight from both strains, where interestingly, the ground samples had shorter doubling times during the log phase than the flight samples.

DISCUSSION

GeneSat – Summary

GeneSat-1 was the first-ever fully autonomous in-situ outer space gene-expression analytical system. It was designed to provide a platform for gene expression experiments using microorganisms that contain green fluorescent protein promoter reporter constructs. The purpose of the initial GeneSat flight, GeneSat-1, was to demonstrate the necessary technology, proving the utility of the hardware for future use in PI-driven flights.

To accomplish this, two strains of *E. coli* constitutively expressing GFP were chosen. The cells were prepared for flight by transferring them to a saline stasis buffer to make them metabolically dormant prior to loading into the satellite hardware and onto the spacecraft. *E. coli* is a well-studied, fully sequenced model microorganism that can be maintained in dormancy for days to months, over a fairly wide temperature range, typically with minimal compromise of future viability and was thus an ideal choice of organism for a first flight. After launch, once the payload reached a stable orbit with minimal forces on the satellite ($< 10^{-3}$ g), the experiment was activated. The culture was fed and brought to a growth temperature of 34 °C.

Once the experiment was initiated, fluorescence and growth measurements were monitored in each well once every 12 minutes. By 10 h after feeding, all biowells exhibited growth and the fastest growing well showed measurable GFP expression. By 20 h after feeding, 6 of 9 biowells exhibited green fluorescence; eventually, the remaining 3 biowells also produced GFP signals. Some of the AcGFP wells were late to express GFP, which was not unexpected because of its mild biocompatibility problem and the prolonged stasis period due to launch slips.

Comparison of the growth and GFP data from flight and ground conditions show a decreased growth rate in the flight. While this may seem to conflict with some of the previously published results (Benoit et al., 2007; Bouloc et al., 1991; Gasset et al., 1994; Kacena et al., 1999; Klaus et al., 1997), most of these results reported higher final growth plateaus in flight and did not measure doubling times per se. Our data also show a trend toward higher final cell numbers in the flight samples; however, this difference in final plateaus was not found to be statistically significant.

GeneSat-1 was a successful mission; cell growth and fluorescence were observed in all biowells, all the data were successfully stored and transmitted, and all parameters remained within the requirements: the temperature remained stable, the fluidic system and optical detectors functioned properly, the sensors indicated that all systems were at nominal status, there was adequate power throughout the experiment to power the heaters, sensors, and communications subsystems. In summary, GeneSat-1 successfully demonstrated a system that is capable of autonomous operation including the necessary environmental control and systems to measure growth and fluorescence for gene expression studies in space.

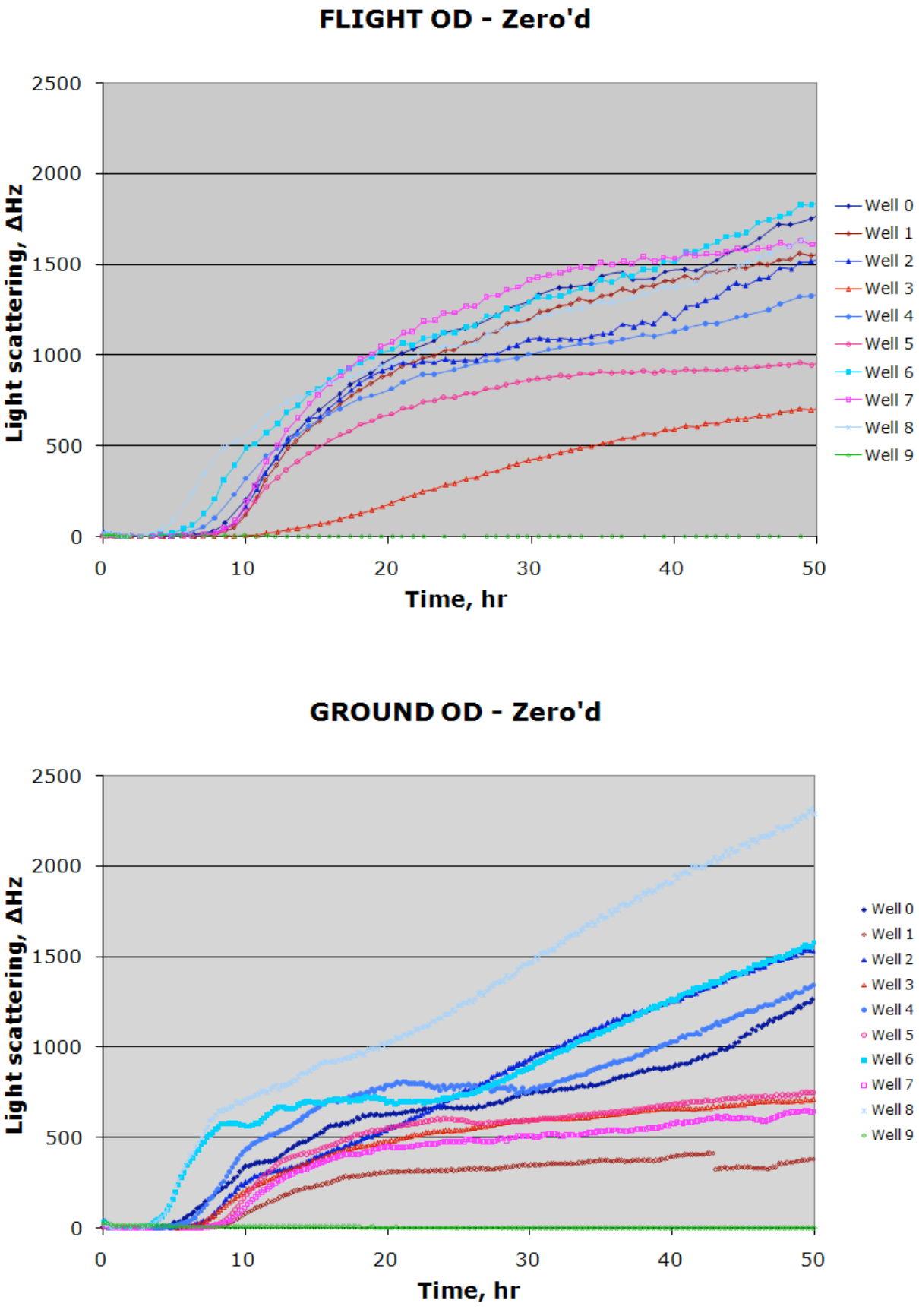

Figure 5: *Normalized optical density readings of flight and ground units*

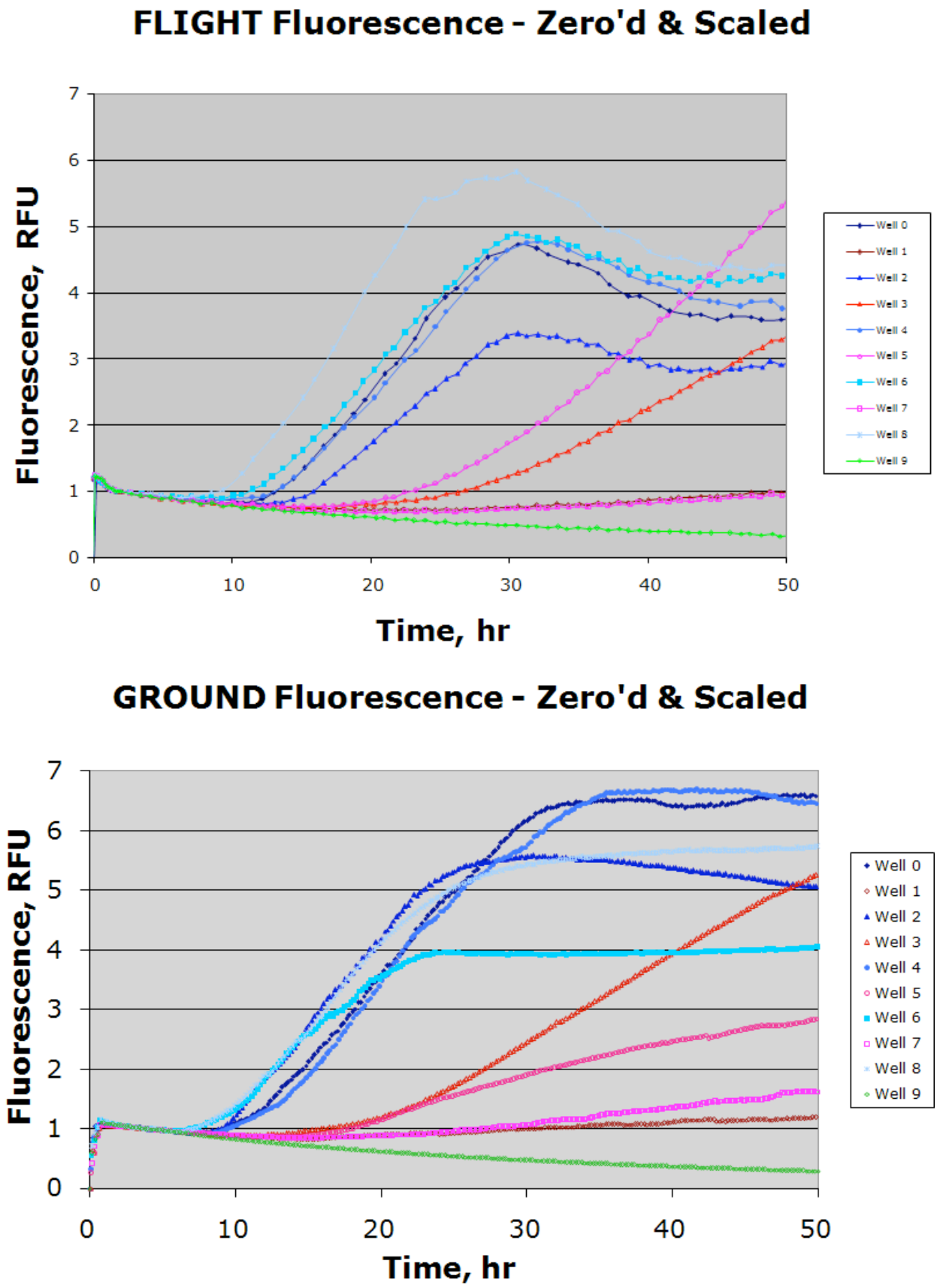

Figure 6: *Normalized fluorescence readings for ground and flight units*

DOUBLING TIMES (minutes) from semi-log PLOTS of OD vs. time

pGREEN

Well No.	*Flight*	*Ground*
0	59	44
2	54	44
4	47	45
6	51	28
8	46	29
Average:	**51**	**38**
SD:	**5**	**8**
	p=0.018	

AcGFP

Well No.	*Flight*	*Ground*
1	44	34
3	41	36
5	48	30
7	47	33
Average:	**45**	**33**
SD:	**3**	**2**
	p=0.0009	

Figure 7: *Calculated doubling times for pGREEN and AcGFP strains*

PharmaSat and Beyond

The successor small satellite project to GeneSat-1, PharmaSat, is a PI-led mission that aims to yield publishable data. Michael McGinnis leads this study of the effects of microgravity on antifungal susceptibility in *S. cerevisiae*. The experiment will test the efficacy of one antifungal agent, voriconazole (VOR), at three different concentrations in flight; results will be compared to a ground control. Yeast cell health will be measured by two methods: optical density to assess overall cell number, and Alamar Blue, a colorimetric indicator of cell viability.

As with GeneSat, cells must be transferred to stasis buffer and loaded in the lab six weeks prior to launch. After launch, when a stable orbit is reached, the experiment will be initiated by starting temperature control (target of 27 °C) followed by feeding the cells with growth media containing Alamar Blue. After allowing the cells to recover from stasis, they will be challenged with the VOR. Growth and viability will be tracked with measurements in each well every 15 minutes for 72 hours. This experiment requires a number of hardware upgrades to the GeneSat system. The fluidic card is being increased in complexity from 10 biowells to 48 biowells comprised of four independent banks of 12 wells each, offering both a larger statistical sampling and the capability for a control plus three independent antifungal concentrations. The off-card fluidic subsystem must allow for two media exchanges and dilutions of the antifungal agent. This dilution process adds complexity to the system, as it must recirculate the liquids for mixing. Finally, the optical system is being changed, resulting in a less complex but much more densely populated system capable of measuring absorbance at three different wavelengths, provided by a dedicated 3-color LED for each biowell.

Figure 8 shows a cross section of the fluidic card. As with GeneSat, the cells are kept inside the well with nylon fiber filters. Unlike GeneSat, which had a gas-permeable membrane only on one side, PharmaSat has gas-permeable membranes on both sides to allow for adequate gas exchange in the "taller" wells.

What's the next development after PharmaSat? Just as PharmaSat increased the hardware complexity relative to GeneSat, the goal is to continue to incrementally increase the complexity of future systems. Planned future developments include: increasing the sample number, adding the ability to handle different and more complex organisms, including sample preparation prior to analysis, decreasing the time between bio-load and launch, and incorporating more complex measurements (luminescence, multi-color fluorescence, imaging fluorescence, etc.). With increasingly complex analytical technology, cells can eventually be processed in orbit for in-flight microarray measurements. The primary thrust with the small-satellite program is to add more complexity and increased capabilities to advance the autonomous possibilities.

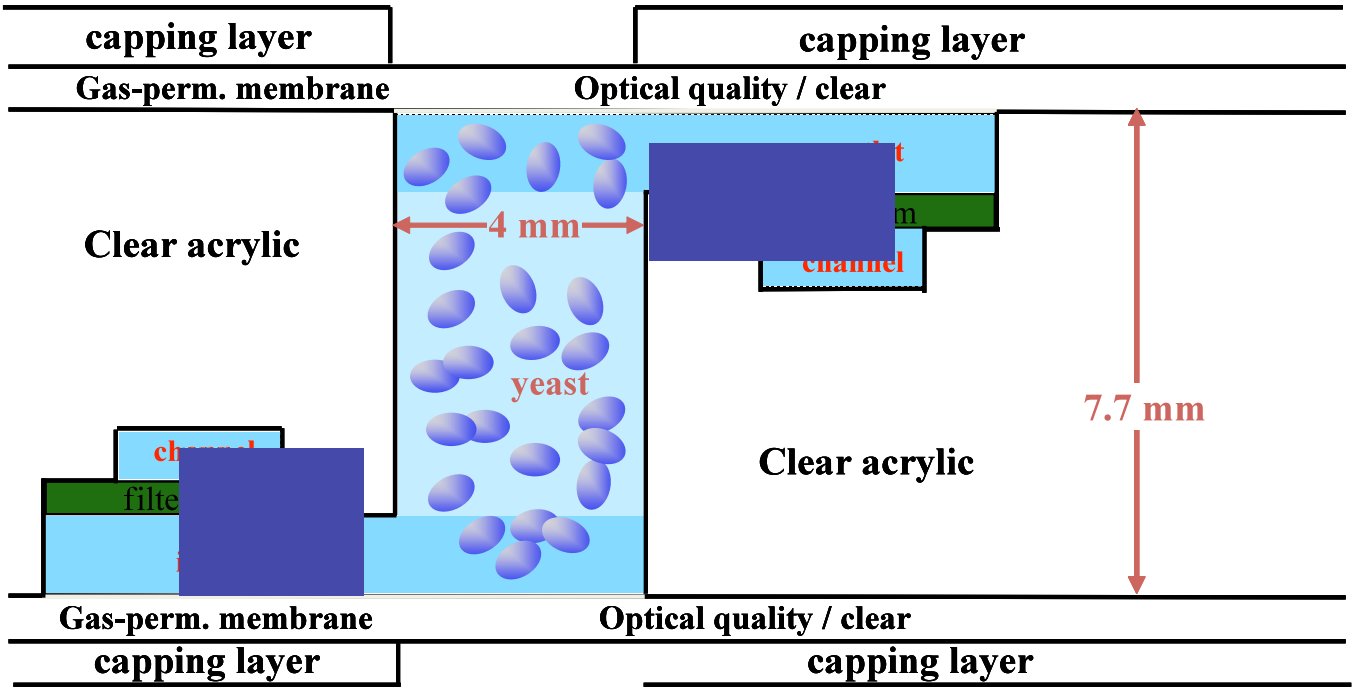

Figure 8: *Cross section of the PharmaSat fluidic card*

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